

Mutation of a *Drosophila* gamma tubulin ring complex subunit encoded by *discs degenerate-4* differentially disrupts centrosomal protein localization

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We have cloned the *Drosophila* gene *discs degenerate-4* (*dd4*) and find that it encodes a component of the γ -tubulin ring complex (γ TuRC) homologous to Spc98 of budding yeast. This provides the first opportunity to study decreased function of a member of the γ -tubulin ring complex, other than γ -tubulin itself, in a metazoan cell. γ -tubulin is no longer at the centrosomes but is dispersed throughout *dd4* cells and yet bipolar metaphase spindles do form, although these have a dramatically decreased density of microtubules. Centrosomin (CNN) remains in broad discrete bodies but only at the focused poles of such spindles, whereas Asp (abnormal spindle protein) is always present at the presumptive minus ends of microtubules, whether or not they are focused. This is consistent with the proposed role of Asp in coordinating the nucleation of mitotic microtubule organizing centers. The centrosome associated protein CP190 is partially lost from the spindle poles in *dd4* cells supporting a weak interaction with γ -tubulin, and the displaced protein accumulates in the vicinity of chromosomes. Electron microscopy indicates not only that the poles of *dd4* cells have irregular amounts of pericentriolar material, but also that they can have abnormal centrioles. In six *dd4* cells subjected to serial sectioning centrioles were missing from one of the two poles. This suggests that in addition to its role in nucleating cytoplasmic and spindle microtubules, the γ TuRC is also essential to the structure of centrioles and the separation of centrosomes.

[Key Words: γ -TuRC; centrosomes; centrioles; mitosis; spindle; microtubules]

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The microtubule (MT) cytoskeleton is an essential and dynamic structure involved in several important physiological events such as cell motility, traffic, signal transduction, apoptosis and cell division (Kelleher and Titus 1998; Gundersen and Cook 1999; Small et al. 1999). The major microtubule organizing center (MTOC) in animal cells is the centrosome, which nucleates the slowly growing minus ends of microtubules allowing the plus ends to extend into the cytoplasm. In most animal cells, centrosomes are essential for definition of the interphase MT arrays, for determination of cell polarity, and for the formation and function of the spindle in mitosis. There are two main components of the centrosome: A pair of centrioles comprising cylinders of nine triplet microtubules and the pericentriolar material (PCM) that appears to provide nucleation centers for cytoplasmic and spindle microtubules. Little is known about the organi-

zation of the PCM, although both pericentrin and γ -tubulin have been described to form a protein complex organized into a lattice like structure (Dicteberg 1998).

γ -tubulin is a conserved member of the tubulin family found at MTOCs, including animal cell centrosomes, and the equivalent organelles of yeasts, the spindle pole bodies (SPBs) (Oakley and Oakley 1989; Horio et al. 1991; Joshi et al. 1992; Sunkel et al. 1995). The γ -tubulin of *S. cerevisiae* forms a 6S complex with Spc98p and Spc97p, associated with both the inner and outer plaques of the SPB (Geissler et al. 1996; Knop et al. 1997). Temperature sensitive mutants of its structural gene, *tub4*, show defects in microtubule nucleation at the newly formed SPB (Marschall et al. 1996) as well as in the assembly of a mitotic spindle (Spang et al. 1996). The *SPC98* gene was identified as a dosage-dependent suppressor of the *tub4-1(ts)* allele (Geissler et al. 1996). Its gene product appears essential for mitotic spindle formation because cells harboring the temperature sensitive allele *spc98-1* or over expressing wild-type protein, duplicate and separate their SPBs but form a defective mitotic spindle

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(Geissler et al. 1996). The gene encoding the other main component of this complex, *SPC97*, was isolated as a suppressor of the *spc9-2(ts)* mutant (Knop et al. 1997). Its temperature sensitive alleles show phenotypes similar to *tub4* and *spc98* mutants as well as defects in SPB duplication (Knop et al. 1997). Spc98p docks the Tub4p complex to the inner plaque of the SPB through the N terminus of Spc110p (Knop et al. 1997; Nguyen et al. 1998), a protein that forms a bridge between the inner and central plaques. Pereira and colleagues (1998) have demonstrated that the Tub4 complex is formed in the cytoplasm. It is transported to the nucleus via the nuclear localization signal (NLS) present in Spc98p (Geissler et al. 1996). Spc98p at the inner plaque of the SPB is phosphorylated in a cell cycle-dependent manner, whereas Spc98p in the outer plaque does not appear to undergo such modification.

In higher eukaryotes γ -tubulin occurs in a 25S–32S complex that has been shown by electron microscopy to have a ring shape leading to the name γ -tubulin ring complex (γ TuRC) (Moritz et al. 1995a; Zheng et al. 1995). The *Xenopus* complex comprises seven proteins: α -, β -, and γ -tubulin, and additional proteins of 75, 109, 133, and 195 kD (Zheng et al. 1995). The 109 kD *Xenopus* protein (Xgrip109) is a homolog of yeast Spc98p (Martin et al. 1998). It interacts directly with γ -tubulin and is essential for microtubule nucleation (Martin et al. 1998). The major human and *Drosophila* γ TuRCs have very similar protein profiles (Moritz et al. 1995a,b, 1998; Zheng et al. 1995; Murphy et al. 1998; Tassin et al. 1998). The 100 and 101 kD human proteins hGCP2 and hGCP3 correspond to Spc97p and Spc98p, respectively (Murphy et al. 1998; Tassin et al. 1998). In *Drosophila*, a second smaller 240 kD γ -tubulin complex has been described (Oegema et al. 1999) comprising only γ -tubulin and the *Sp97/98* homolog *Dgrip84* and *Dgrip91*. It is proposed that this is assembled into the complete 3 MDa γ -TuRC, which contains multiple copies of the heterotrimer plus ancillary proteins.

A series of biochemical studies have shown that the γ TuRC is required for the assembly of the centrosome around sperm centrioles in *Xenopus* egg extracts (Félix et al. 1994; Stearns and Kirschner 1994) and that it is required for MT nucleation in vitro (Moritz et al. 1995a; Zheng et al. 1995; Oegema et al. 1999). In vitro assays have shown that the γ -TuRC is essential for the MT nucleation properties of centrosomes from sea urchins and *Drosophila* (Moritz et al. 1998; Schnackenberg et al. 1998). In these assays microtubule nucleation is reconstituted from two components: inactive salt-stripped centrosome scaffolds and a high speed cytoplasmic supernatant. These assays have been used in *Drosophila* to show that, although γ -tubulin is a necessary component of the cytoplasmic fraction, it is insufficient, and that MT nucleation also requires the abnormal spindle protein (Asp) (Moritz et al. 1998; Avides and Glover 1999). However, other known centrosome-associated proteins, CP60 and CP190 are not required for MT nucleation.

This leads to a model of the γ TuRC in *Drosophila* in which the complex is assembled in the cytoplasm from

the heterotrimer subunits and recruited onto the centrosome, where it nucleates microtubules (Moritz et al. 1998; Oegema et al. 1999). In mitosis it functions in concert with Asp, or a protein with equivalent function in other organisms, to organize the spindle microtubules (Avides and Glover 1999). This implies that the centrosome might be essential for MT nucleation and therefore for the formation of spindles. However, in the female meiotic divisions of *Drosophila* the spindles form in the absence of centrioles (Riparbelli and Callaini 1996) and without detectable concentration of γ -tubulin at the poles (Debec et al. 1995; Tavosanis et al. 1997). Moreover, loss of centrosomes has been observed from the spindle poles in the syncytial embryos of several mutants, and although these generally lead to the accumulation of mitotic defects, several rounds of mitosis can take place on such spindles (Gonzalez et al. 1990; Glover et al. 1995; Megraw et al. 1999; Vaizel-Ohayon and Schejter 1999). These observations, together with the ability to build spindles without centrosomes in vitro that are able to undertake metaphase and anaphase, has been taken to mean that centrosomes might be dispensable in the formation of a functional spindle in some systems. Recent observations that a functional spindle can still form in mammalian cells after laser ablation of the centrosomes (Khodjakov et al. 2000) now reinforce this idea.

One route toward understanding the role of the centrosome is to study the consequences of defects in its individual components within animal cells that obey checkpoint regulatory mechanisms often absent in embryonic systems. In this paper, we use a genetic approach to illustrate in vivo the effects of disrupting the γ -TuRC in *Drosophila*. We have characterized at the molecular level the locus of *l(1)discs degenerate-4* (*dd4*), an essential gene for progression through mitosis that we show encodes the Spc98p homolog, *Dgrip91*. We show here that mutations in *dd4* disrupt the localization of γ -tubulin to the centrosome, and also differentially effect the distribution of other centrosomal proteins; as a consequence, a spindle is formed that enables mitosis to proceed to a metaphase-like state, but in which anaphase progression is severely delayed or abolished.

Results

Mutations in dd4 delay progression through metaphase

The first mutants of the X-linked gene *dd4* were identified as having missing or degenerate discs in third instar larvae (Stewart et al. 1972); it was subsequently shown that neuroblasts have an elevated mitotic index and a low proportion of anaphase figures (Gatti and Baker 1989). We examined the mitotic defects in larval brains from males of several mutant alleles (see Materials and Methods) and observed three striking phenotypes. First, the mitotic index is elevated five- to sixfold in each allele (Fig. 1F, Table 1). Second, many cells contain polyploid nuclei, the proportion of which increases in relation to the severity of the lethal phase of the allele. Finally, a

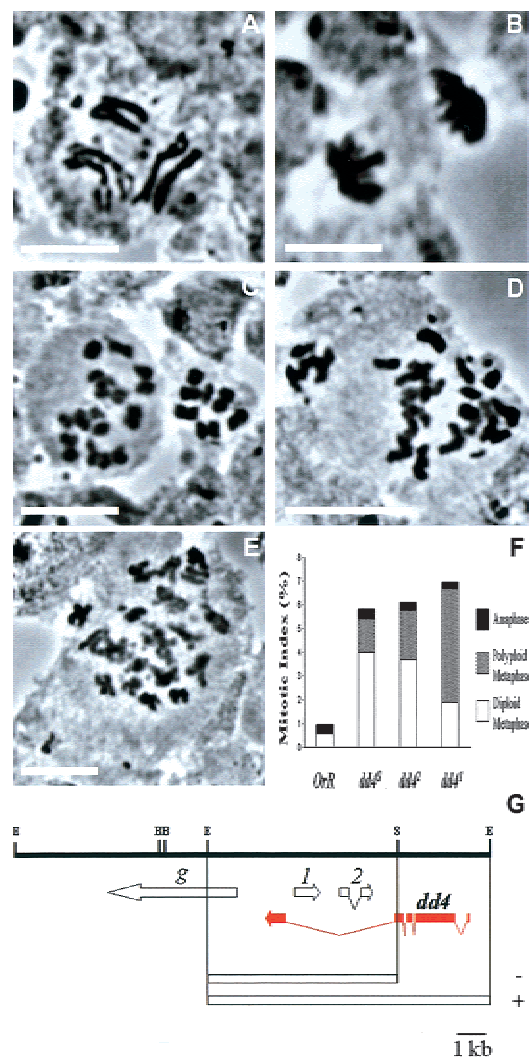


Figure 1. Mitotic defects revealed in orcein-stained preparations of the CNS of *dd4¹* larvae. Panels A and B show respective metaphase and anaphase figures from wild-type female larvae. Panels C–E are from *dd4¹* mutant male larvae. Note the extreme chromatin condensation of mutant cells compared to the wild-type cells. Euploid (right) and 8N (left) metaphase cells (C). (D) An anaphase cell. We classify this cell as an anaphase as many sister chromatids appear to have separated and some appear as if pulled to poles. These cells are in fact rather difficult to classify, but resemble the catastrophic anaphases reported by Gatti and Baker (1989) for this mutant allele. (E) A 16N metaphase cell. (F) Proportions of euploid and polyploid metaphases and anaphase figures in three *dd4* alleles in comparison to the wild-type strain Oregon-R. (G) Molecular organization of the *dd4* gene. (g) *garnet*. The *dd4* locus in the 12B5 region is contained within a 9kb *EcoRI* (E) fragment (+) that shows altered mobility in *xx16* (see Materials and Methods). This restriction fragment contains also two other open reading frames: One with similarity to a variety of sugar isomerases (transcript 1) and another similar to rat estradiol 17 β -dehydrogenase (transcript 2). The 9 kb fragment and the indicated sub-segment of it (–) were introduced into germ-line cells by P-element mediated transformation. Transformant lines carrying the + fragment were able to rescue the phenotypes of all three *dd4* alleles tested, whereas those carrying the – fragment in which the *dd4* gene was truncated could not. Bar, 20 μ m.

further striking feature of the *dd4* mutant mitotic figures is the degree to which metaphase chromosomes are overcondensed (Fig. 1C,E) compared to wild-type (Fig. 1A). It appears that cells are able to pass through the strong metaphase delay and undergo multiple rounds of S phase because, in addition to 4N mitotic cells (right hand cell in Fig. 1C), it is possible to see 8N (left hand cell in Fig. 1C) and 16N cells (Fig. 1E). The anaphase figures frequently appear to have disorganized arrays of chromosomes (Fig. 1D) and broad spindle poles when compared to wild type (Fig. 1B).

The range of severity of the mitotic phenotype and the lethal phases of males places the different *dd4* alleles into a series. To determine the extent of hypomorphs of these mutant alleles, we made heterozygous females between the weakest allele, *dd4^S*, and each member of the series together with a deficiency for the chromosomal region in which *dd4* lies. We scored their mitotic phenotype in larval brain cells, as well as their viability and ability to produce eggs. In this analysis we also included a new mutant chromosome carrying a *dd4* allele generated by X-ray mutagenesis which we were unable to analyze in males as it carries another lethal mutation (see Materials and Methods). The severity of the phenotype shows the progression: *dd4^S* < *dd4²* = *dd4^{xx16}* < *dd4¹* < *Df* (Table 2). As a stronger phenotype is seen when *dd4^S* is placed against the deficiency than when against *dd4¹*, we conclude that *dd4¹* is not amorphic for this aspect of phenotype. The lethal phases of these allelic combinations fall into the same series, except that both *dd4^S*/*dd4¹* and *dd4^S*/*Df* appear similarly severe in that females rarely eclose with the few emerging adults having extreme cuticular defects. Only the weakest allelic combinations produce more than the occasional egg, and while these appear to have both maternally and paternally derived chromosomes, they fail to develop.

Cloning of *dd4* reveals it encodes the 91 kD component of the γ -tubulin ring complex

To begin to understand the biochemical basis for the mitotic defects in *dd4* mutants, we cloned the gene using a positional cloning strategy. *dd4* had been shown to be tightly linked to two genes in region 12B on the X-chromosome, *mus101* and *garnet* and is uncovered by the deficiency *Df(1)wLCD* (Axton 1990; Yamamoto et al. 2000; A. Schalet, pers. comm.). We had constructed a detailed molecular map of this region, following a ~150 kb chromosome walk, on which we had identified the locations of both *mus101* and *garnet* (Yamamoto et al. 2000). The position of *dd4* on this map, proximal to *garnet*, was first revealed through the recovery of the X-ray-induced lethal allele, *dd4^{xx16}*. This mutant failed to complement *dd4^S* and altered the size of the 9 kb *EcoRI* restriction fragment that contained the very 5' end of *garnet*. This result therefore established a physical linkage between *garnet* and *dd4* of <9 kb.

The corresponding wild-type restriction fragment was sequenced in its entirety and used to isolate cDNAs that were also sequenced (see Materials and Methods). This

Table 1. Quantification of mitotic activity in wild type and dd4 mutant neuroblasts

Genotypes	No. of cells ^a	Metaphases/%				Anaphases/%	Mitotic Index ^b /%	Metaphase: Anaphase
		diploid	tetraploid	polyploid	total			
OrR	9066	0.60	0	0	0.60	0.38	0.97	1.6
dd4 ¹ /Y	5605	1.9	0.34	4.4	6.6	0.27	6.9	24
dd4 ² /Y	6005	3.7	0.37	1.7	5.8	0.30	6.1	19
dd4 ^S /Y	7102	4.0	0.07	1.3	5.5	0.42	5.9	13

^aTotal number of cells scored from each genotype. Ten fields were observed per brain and five brains were dissected for each genotype.

^bThe Mitotic Index is given here as the percentage of the total number of cells from each genotype.

enabled us to identify three genes in this region in addition to *garnet*: One encodes an enzyme similar to a variety of sugar isomerases (Fig. 1G, gene 1); another encodes an enzyme similar to murine estradiol 17 β -dehydrogenase (Fig. 1G, gene 2); and the third encodes a protein homologous to *S. cerevisiae* spindle pole body protein Spc98 and its homolog in human (hGCP3) and *Xenopus* (Xgrip109) (Fig. 1G, *dd4*). Moreover, genes 1 and 2 were found to be nested within the *SPC98* homolog. Of these three genes, the latter was the best candidate for *dd4*, as mutation of a centrosomal component might be expected to result in the broad spindle poles seen in *dd4* cells (see above). To prove this identity we carried out germ-line transformation experiments using two constructs: One predicted to contain the complete putative *dd4* transcription unit, and a corresponding negative control in which the promoter and first four exons of the putative *dd4* gene were deleted, but which retained the two nested genes (Fig. 1G). We found that the construct carrying the entire *SPC98* homolog fully rescued the lethal phenotype of *dd4* alleles, whereas the truncated version did not. This established that *dd4* is a structural homolog of yeast *SPC98* and its vertebrate counterparts. Another group has recently and independently identified this gene as encoding a component of the γ -TuRC, which they have named *Dgrip91* (Oegema et al. 1999).

The sequence of *dd4/Dgrip91* revealed the gene to contain five exons encoding a 923-amino-acid protein. *Dgrip91* is more related to hGCP3 and Xgrip109 than to Spc98. In each of these organisms, Spc98 or its counter-

parts is one of a pair of related proteins. We searched the *Drosophila* genome database for the sequence of the other member (*Dgrip84*) of this pair of genes. We find that in *Drosophila*, as in human hGCP2 and hGCP3 (Murphy et al. 1998) *Dgrip84* and *Dgrip91* are more related to each other than to either of their yeast homologs.

γ -tubulin is dispersed but centrosomin remains in discrete bodies in dd4 mutants

Because *dd4* encodes a component of the γ -tubulin ring complex, we examined the organization of the mitotic spindle and its poles in cells from the central nervous system of *dd4* mutants. We first examined the localization of γ -tubulin in relation to centrosomin (CNN), another component of the *Drosophila* centrosome first described by Heuer et al. (1995). In mitotic cells from wild-type brains, these two proteins colocalize to the two centrosomes (Fig. 2A). In cells of all three *dd4* mutant alleles, γ -tubulin staining can still be detected; however, it is no longer found in a well-defined body but rather is distributed throughout the cell (Fig. 2B,C). In contrast, distinct CNN-containing bodies can be seen in every mitotic cell. However, whereas wild-type cells invariably contained two such bodies, the functional centrosomes, some mutant cells contained only one body stained with CNN (Fig. 2B), while others contained two (Fig. 2C).

In cells stained to reveal the spindle microtubules, we found that the CNN-containing bodies always appeared

Table 2. Mitotic activity in neuroblasts from females with different inter-allelic combinations of dd4

Genotypes	No. of cells ^a	Metaphases/%			Anaphases/ total	Mitotic Index ^b /%
		diploid	polyploid	total		
dd4/+ ^c	5808	0.95	0	0.95	0.31	1.3
dd4 ^S /dd4 ^S	4314	2.7	0.32	3.0	0.23	3.2
dd4 ² /dd4 ^S	2169	4.0	0.78	4.7	0.14	5.0
dd4 ¹ /dd4 ^S	4408	5.9	0.70	6.6	0.02	6.6
dd4XR16/dd4 ^S	3549	4.4	0.76	5.2	0.14	5.3
Df(1)LCD/dd4 ^S	2139	6.9	0.84	7.8	0.05	7.8

^aTotal number of cells scored from each genotype. Ten fields were observed per brain and three to five brains were dissected for each genotype.

^bThe Mitotic Index is given here as the percentage of the total number of cells from each genotype.

^cWeighted average of two Df(1)LCD/FM7, one *dd4^S/FM7*, one *dd4^S/Binsn*, one *dd4^L/Binsn*, and one *dd4^{XR16}/FM7* brains. All these control animals were siblings of the tested mutant larvae.

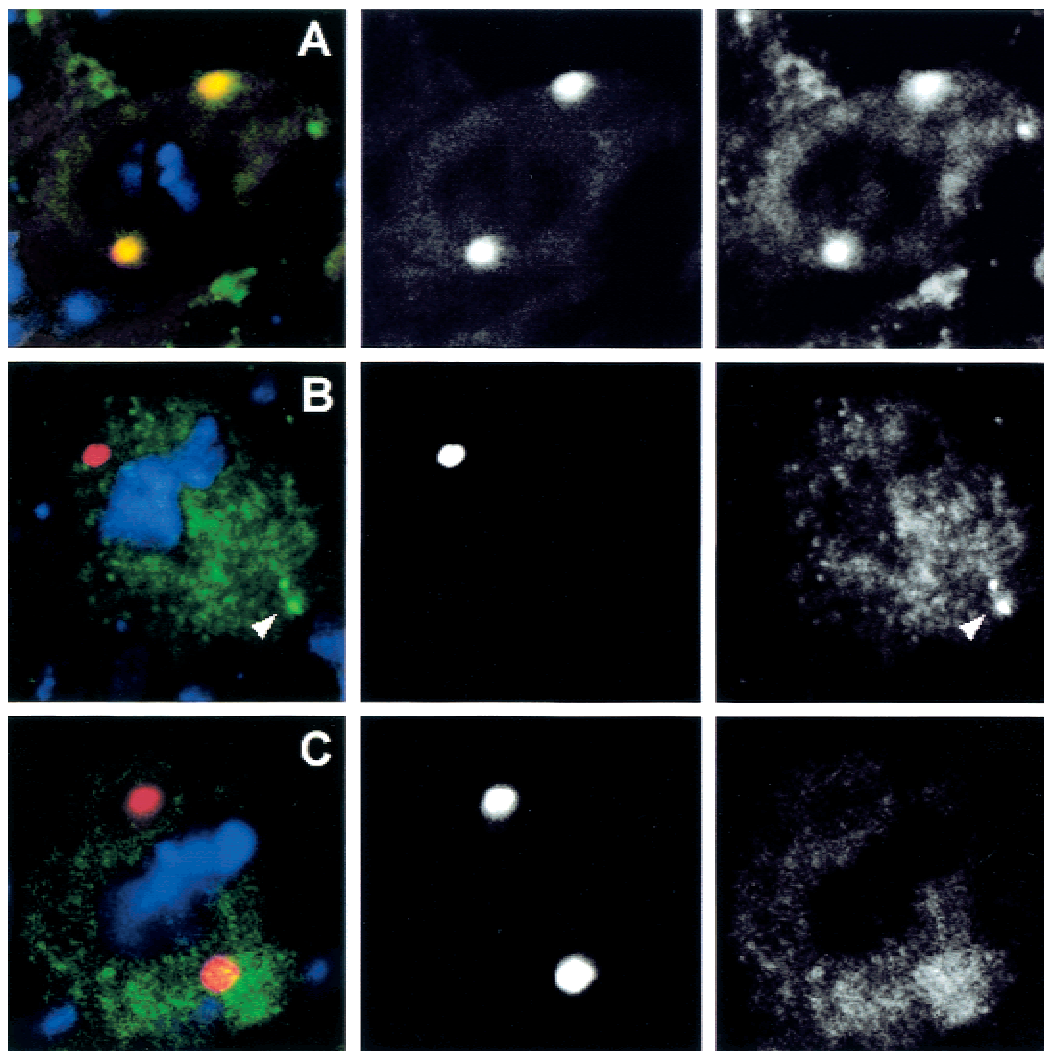


Figure 2. Localization of CNN and γ -tubulin. In each row, a merged image is shown in the left hand panel showing DNA (blue), CNN (red), and γ -tubulin (green). The individual channels showing CNN and γ -tubulin staining are shown in the center and on the right hand side, respectively. (A) A wild-type cell in metaphase showing colocalization (yellow) of CNN and γ -tubulin at the spindle poles. (B) Mitotic cell from a *dd4*¹ larva showing dispersed γ -tubulin with a focus of CNN at one pole but none at the other (arrowhead). (C) Cell from a *dd4*² larva showing dispersed γ -tubulin but well-defined CNN containing bodies at both poles. The centrosome–centrosome distance in panel C is 10 μ m. All panels are shown at the same magnification.

to be associated with a microtubule organizing center. In wild-type cells, these are spherical centrosomes at the spindle poles and astral microtubules (Fig. 3A). In the *dd4* mutant cells, the CNN staining bodies were often less tightly defined structures (see the pole bodies marked by the arrowheads in Fig. 3B–D) and astral microtubules were not seen. In some cells the CNN containing body appeared to have fragmented, and a satellite body could be seen near the main pole (Fig. 3C, arrow). In those cells having only one CNN-staining body, prominent arrays of microtubules extended between this pole and the chromosomes (Fig. 3D). Conversely, microtubules extending from the chromosomes to the pole lacking the CNN body exhibited reduced staining (Fig. 3D), and in some cells this pole appeared not to have organized microtubules.

Punctate bodies containing Asp are found at the ends of dispersed microtubules

In addition to the γ -tubulin ring complex, the Asp protein is also known to be required to nucleate asters of microtubules (Avides and Glover 1999). We therefore wished to know the consequences of disrupting the γ -tubulin ring complex upon the localization of Asp protein. In wild-type mitotic cells, the Asp protein is found on the face of the centrosome that makes contact with spindle microtubules. This can be seen in relationship to the localization of γ -tubulin in the centrosome in Figure 4 (panel A). This close juxtaposition of γ -tubulin and Asp is no longer seen in *dd4* cells in which the γ -tubulin is dispersed, but Asp maintains a punctate distribution. This punctate staining can be clustered around the

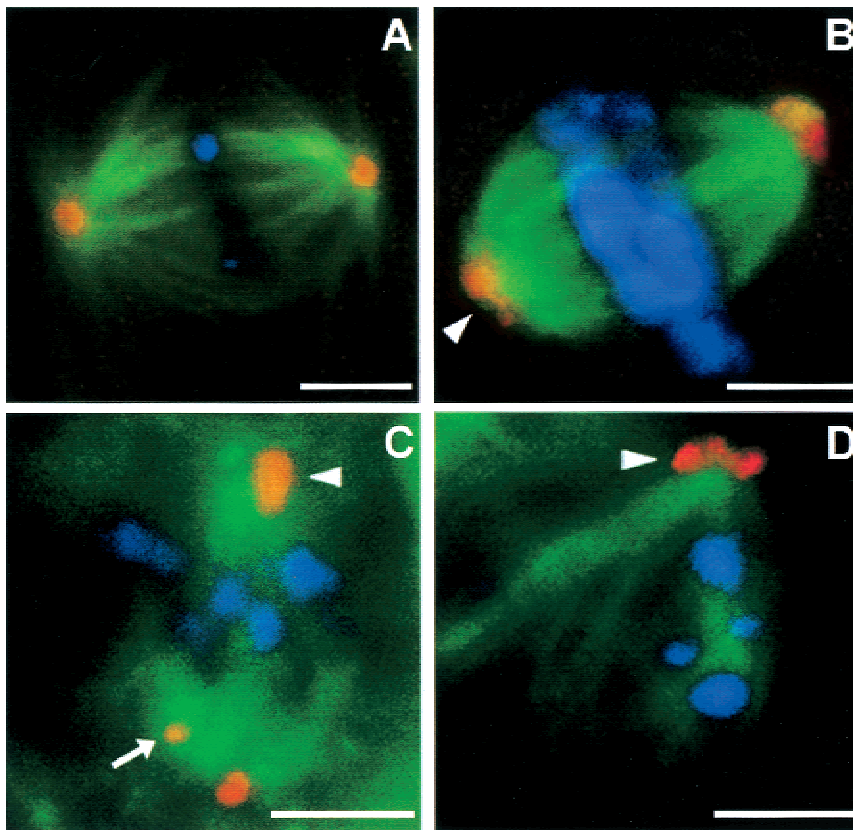


Figure 3. Localization of CNN with respect to spindle microtubules. Merged images showing DNA (blue), α -tubulin (green), and CNN (red). (A) A wild-type cell in metaphase. (B) A *dd4^S* cell showing a broader distribution of CNN at the poles of the spindle (arrowhead). (C) A *dd4^I* cell with evident defects in CNN organization at the poles. One broad spindle pole (arrowhead) and an ectopic organizing center at the opposite pole indicated by the arrow. (D) A *dd4²* cell with one well organized spindle pole showing presence of CNN (arrowhead) and one disorganized pole lacking CNN. Bar, 5 μ m.

spindle poles or clustered in one area and scattered throughout the remaining part of the cell (Fig. 4B, arrows), but is never diffuse as is γ -tubulin. Immunostaining to reveal microtubules shows that Asp protein is always found at the poles of bipolar spindles, either as a well organized body, but more usually in clustered aggregates (pole marked with arrowhead in Fig. 5B). In spindles that had only one focused pole, individual bundles of the microtubules could be seen to extend both from this focus and from small punctate caps of Asp at the unfocused pole towards the chromosomes (Fig. 5C,D, arrows).

The CP190 antigen accumulates around chromosomes in dd4 mutants

CP190 is an abundant protein which, together with its partner CP60, associates with mitotic centrosomes in *Drosophila* cells (Kellogg et al. 1995; Whitfield et al. 1995). Although frequently used as a marker to follow centrosome behavior, its function remains unknown. In wild-type cells, CP190 is found associated with the centrosomes at the spindle poles (Fig. 6A). In the weakest allele, *dd4^S*, some of the antigen is lost from the poles and appears in the central part of the spindle in the region occupied by the mitotic chromosomes (Fig. 6B). In the stronger alleles, only a small proportion of CP190 remains at the poles; the remainder is clustered around the chromosomes (Fig. 6C,D). Thus, intact γ -tubulin ring

complex appears to be required at the poles in order to maintain the polar association of the CP190 protein.

The organization of centrioles is disrupted within dd4 centrosomes

In addition to being present in the PCM, γ -tubulin is also found within the centrioles themselves and appears to be required for centriolar function (Ruiz et al. 1999; Marshall and Rosenbaum 2000). To determine whether *dd4*

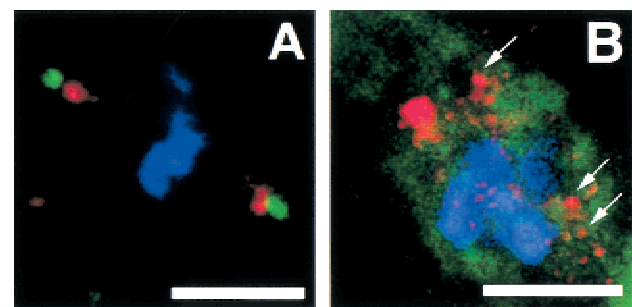


Figure 4. Localization of Asp and γ -tubulin. Merged images showing the distribution of DNA (blue), Asp (red), and γ -tubulin (green). (A) Wild-type cell at metaphase showing the localization of Asp on the side of the centrosome facing the metaphase plate. (B) A *dd4^I* cell with one pronounced aggregate of Asp and multiple punctate Asp bodies (arrows). γ -Tubulin is also dispersed in this cell. Bar, 5 μ m.

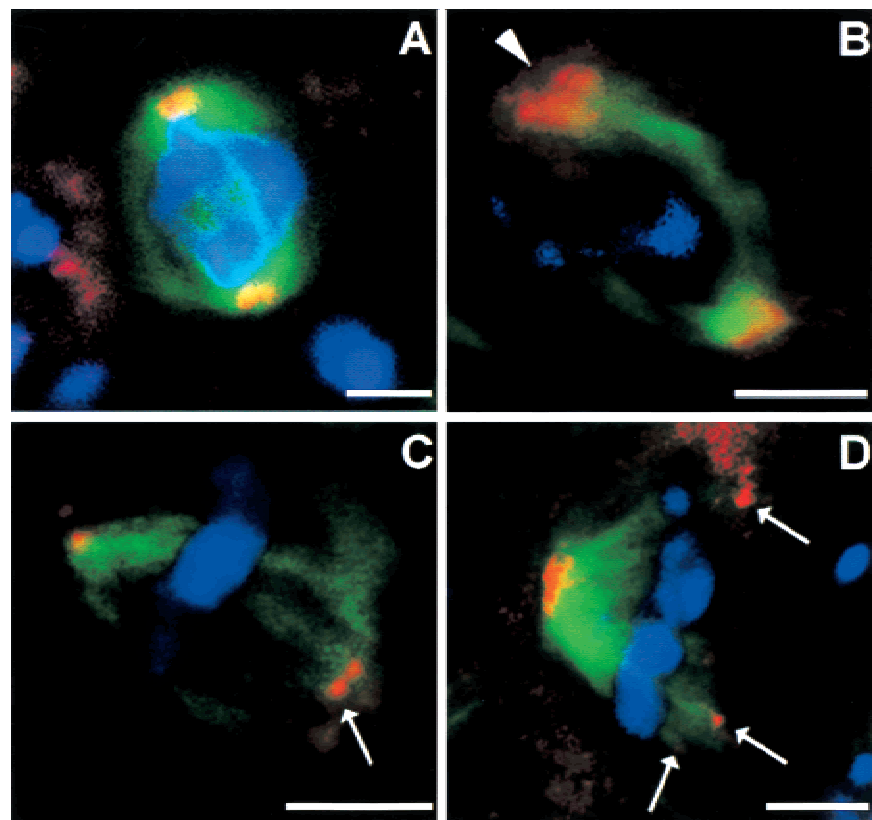


Figure 5. Localization of Asp with respect to spindle microtubules. Merged images showing DNA (blue), Asp (red), and α -tubulin (green). (A) A wild-type cell in anaphase. (B) A $dd4^1$ cell showing mislocalization of Asp around the pole of the less organized half of the spindle (arrowhead). (C,D) Asp contacting the tips of microtubules (arrows) in the abnormally broad spindle poles of $dd4^2$ cells. Bar, 5 μ m.

mutants showed any irregularity of centriolar structure, we examined the ultrastructure of centrosomes by electron microscopy of serial sections of cells from the larval central nervous system (photographs kindly provided by Adelaide Carpenter, University of Cambridge, UK). These data consist of complete sets of serial sections through some four wild-type prometaphase cells with chromosomes undergoing congression, one wild-type cell at metaphase, and six $dd4^1$ mutant cells that are in a metaphase-like state. The major features of the ultrastructure of the mitotic spindle of a wild-type cell are indicated in the two consecutive sections of the same cell displayed in Figure 7 (panels A and B). The centrosomes have well defined centrioles showing typical arrays of triplet microtubules surrounded by the electron-dense PCM (Fig. 7A,B, circled in red). Arrays of spindle microtubules (Fig. 7A,B, green tracing) extend from these centrosomes towards condensed chromosomes (outlined in blue). These microtubules occur in bundles that make contact with well defined kinetochore plates on the chromosomes (Fig. 7A). All of the $dd4$ cells examined had two opposing poles, generally broad, but secondary poles were often present. Centrioles were present only at one of the two main poles in all six of the cells, but there were up to four of them; they varied in size and were not consistently arranged as perpendicular pairs. The amount of PCM varied from one pole to another showing no correlation with the pattern of centrioles. Two sections from the same $dd4$ cell are shown in Figure 7 at the planes of each of its spindle poles separated by 0.63 μ m (Fig. 7C,D, circled in red). One pole has an irregular elec-

tron dense region of PCM that surrounds three centrioles, two of which are visible in this section. These two

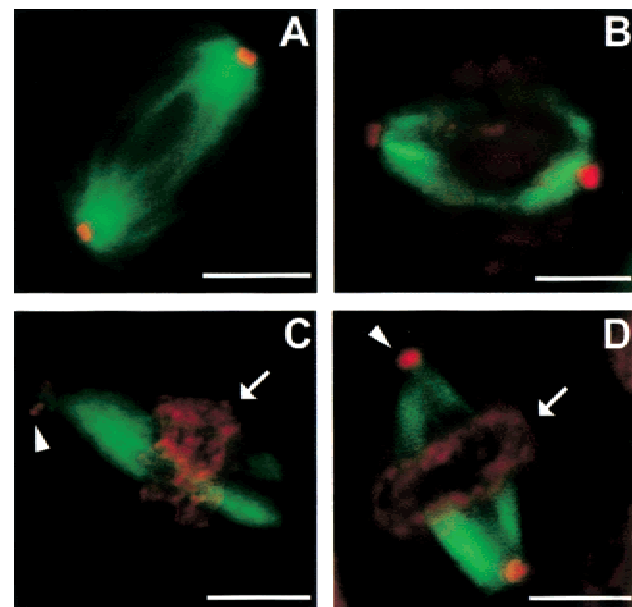


Figure 6. Localization of CP190. Merged images showing CP190 (red) and α -tubulin (green). (A) A wild-type cell at anaphase showing CP190 concentrated in the centrosomes. (B) A $dd4^S$ cell similar in appearance to wild-type but with a trace of CP190 in the central part of the spindle. (C,D) $dd4^1$ neuroblasts with weaker CP190 at the poles (arrowhead in C) and clustering of the antigen around chromosomes (arrows). Bar, 5 μ m.

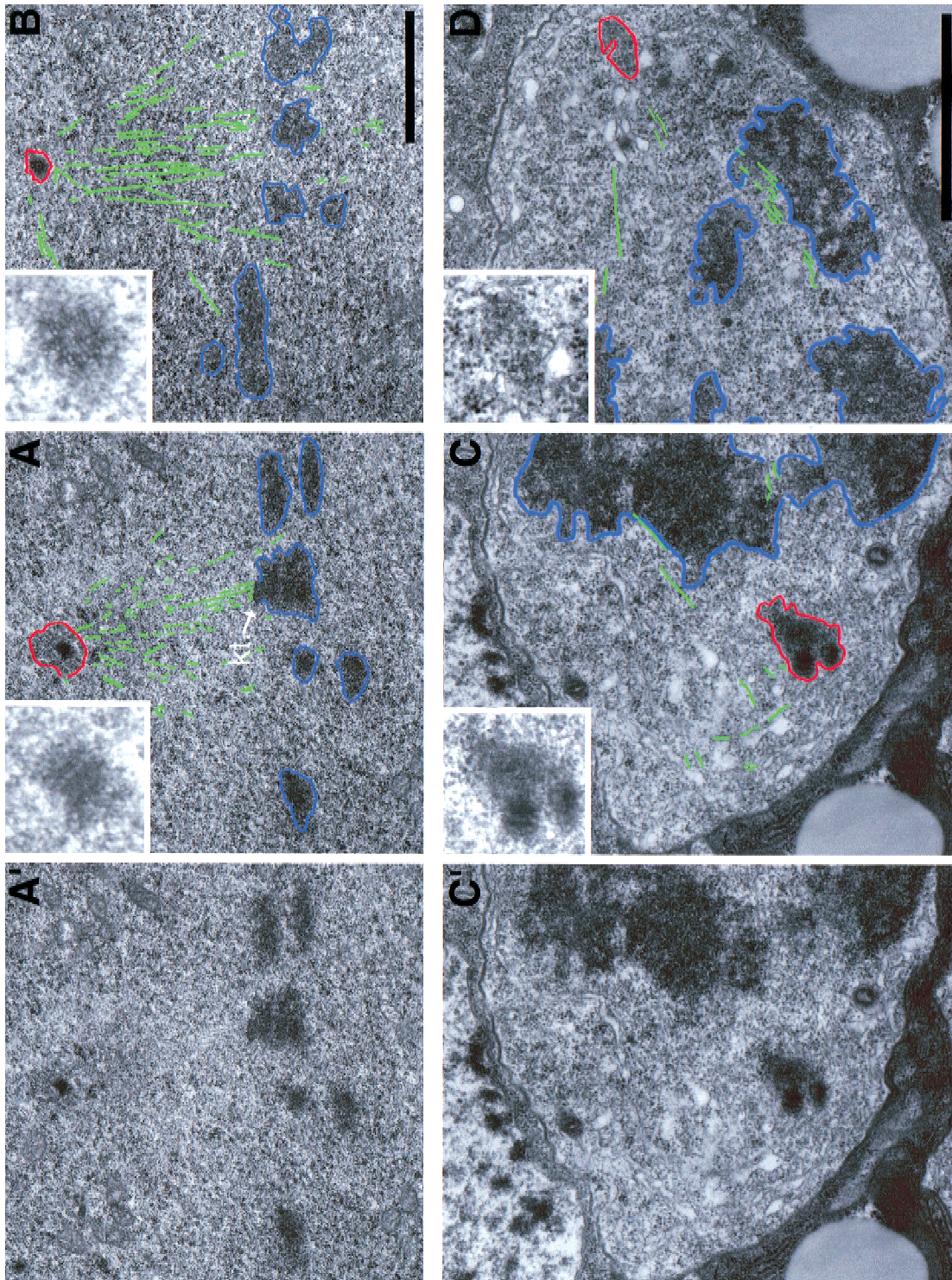


Figure 7. Electron micrographs of spindle poles from wild-type and *dd4* mutant cells. In these micrographs, the PCM of the centrosome has been outlined in red and is also shown at higher magnification in the insets. Microtubules have been traced in green and the outlines of centrosomes in blue. Untraced sections (A', C') are shown of the same micrographs as those with tracings in panels A and C. (A, B). Two adjacent sections 90 nm thick from a wild-type metaphase cell showing the centrosome at one pole. The typical triplet microtubules can be seen in the inset panels. In panel A a kinetochore can be seen on one of the chromosomes [kt] where it makes contact with a bundle of microtubules. Note the alignment of chromosomes on the metaphase plate. (C, D) The *dd4* cell represented in these two panels was sectioned nearly perpendicular to the plane of its spindle. Panel C shows one of its poles, and panel D, the other. The two poles are seven sections apart (630 nm), and thus the continuity of two of the centrosomes can be seen between the two panels. The chromosomes are not aligned as in the wild-type metaphase cell. Note the low density of microtubules and the presence of a defective centriole at one pole [C, inset], and the absence of a centriole from the other (D, inset). Bar, 1.8 μm.

are comparable in structure to wild type, whereas the third is substantially shorter. None are distributed in the usual orthogonal arrangement (Fig. 7C). There appear to be three foci towards which microtubules converge at this pole (sections not shown). A less coherent cluster of PCM can be seen at the second pole, which completely lacks centrioles. The density of spindle microtubules in this and the other *dd4* cells is strikingly reduced compared to the wild-type spindle (Fig. 7C,D, green tracings), consistent with the impression gained from immunostaining (Figs. 3,5,6). The spindle microtubules made poor contact with the mitotic chromosomes and, in contrast to wild-type cells, it was difficult to see organized plate-like kinetochores on *dd4* chromosomes.

Discussion

Our identification of the *Drosophila dd4* gene as encoding a homolog of the budding yeast Spc98 protein has provided the first opportunity to study disrupted function of a member of the γ -tubulin ring complex, other than γ -tubulin itself, in a metazoan cell. It is therefore of interest to compare phenotypes of mutations in *dd4* with mutations in the γ -tubulin genes. *Drosophila* has two genes for γ -tubulin; the one at 23C is expressed in a variety of tissues including brains, imaginal discs and testes, whereas expression of the second at 37C is restricted to ovaries and embryos (Sunkel et al. 1995; Tavosanis et al. 1997). Like the *dd4* mutants, cells from γ -*tub23C* brains display abnormally high levels of chromosome condensation, spindles with defective or absent poles, and polyploidy. However, whereas the mitotic index of *dd4* cells is dramatically elevated, the mitotic index of γ -*tub23C* cells is reduced relative to wild-type and anaphase figures are very rare (Sunkel et al. 1995). The reasons for these differences are not clear. It is as though reduction in levels of the 23C γ -tubulin lead to a limited numbers of chromosome duplication cycles in the absence of mitosis, but this is followed by interphase cell cycle arrest. In contrast, the dramatic increase in metaphase figures in *dd4* mutants resembles a more typical spindle checkpoint arrest. The frequency of anaphases, not dissimilar in total number to wild type, suggests that cells can evade this checkpoint at some frequency as has been described in several organisms. The phenotypes of various allelic combinations suggest that the strongest allele, *dd4*¹, is not completely amorphic by genetic criteria. Western blotting indicated that there is 80%–90% reduction of the Dgrip91 protein in this allele (data not shown). Although we cannot be certain whether this residual protein has any function, this and the genetic observations suggest there may be some residual function of the γ -TuRC even in the *dd4*¹ mutant.

It is generally thought that the late survival of larvae with extreme mitotic defects reflects perdurance of maternal contribution to the oocyte from the heterozygous mother, as has been shown for other cell-cycle genes (Baker et al. 1978). In the case of *dd4*, this assumption has been challenged by the report of normal oocytes from homozygous *dd4*¹ mitotic ovarian clones arising in het-

erozygous females (Perrimon et al. 1989). However, our preliminary observations of embryonic development in eggs produced from allelic combinations weak enough to give viable escaper females (*dd4*^S/*dd4*^S and *dd4*^S/*dd4*³) do indicate that there is a vital maternal contribution to the oocyte: Such eggs appear to have parental DNA but they fail to undergo any development. These observations, which will be reported in detail elsewhere, suggest that if mothers carrying weak enough allelic combinations to be compatible with survival to adulthood cannot build a viable egg, then either the observations of Perrimon and colleagues are in error, or the observed clones had sufficient perdurance of the wild-type product to build eggs indistinguishable from normal heterozygotes.

Together Dgrip84, Dgrip91, and γ -tubulin form the three major components of the γ -TuRC and are homologous to the budding yeast proteins Spc97, Spc98, and Tub4. Genetic and molecular studies show interactions between these genes in budding yeast, and their requirement for SPB structure, duplication, and separation (Geissler et al. 1996; Knop et al. 1997). Interactions between members of this complex and other components of the SPB and spindle are only beginning to be understood. Spc98, for example, binds to the N-terminal region of Spc110p, a coiled-coil protein that spans between the inner and central plaques of the SPB (Sundberg and Davis 1997; Nguyen et al. 1998). The calmodulin binding C terminus of this protein contacts the central plaque and the N-terminal region, the inner plaque. Thus Spc98 might form an essential link between Spc110 and the spindle microtubules that emanate from the inner plaque and the defective spindle structures seen in *spc98* mutants may be a direct consequence of defects in this interaction. The phenotypes of *spc98* mutants thus have some parallels with *dd4* mutants in abnormal spindle microtubule density and organization, and it will be of interest to determine whether Dgrip91 has similar interactions with specific components of the centrosome.

The more drastic disruption of purified preparations of centrosomes with KI in vitro removes a set of proteins, including the γ -TuRC, CP60, CP190, CNN, and Asp, thus destroying their ability to organize microtubules (Moritz et al. 1998; Avides and Glover 1999). The salt treatment appears to leave behind unidentified core centrosomal components since the structure of the PCM is changed very little when examined by electron microscopy. In contrast to salt extraction, reduction of functional Dgrip91 has a differential effect upon the loss of centrosomal antigens. CNN remains in distinct bodies at most of the well focused poles indicating that its centrosomal association is not dependent upon the presence of the γ -TuRC. The defects of *centrosomin* (*cnn*) mutants have been characterized for a number of alleles that show maternal effects and male sterility. These indicate that its function is required for the integrity of both centrosomal and centriolar structures (Li et al. 1998; Megraw et al. 1999; Vaizel-Ohayon and Schejter 1999). Syncytial embryos derived from *centrosomin* mutant mothers undertake up to 12 rounds of mitosis upon spindles whose poles have very little or none of the cen-

trosomal proteins CP60, CP190, or γ -tubulin, and have no astral microtubules. Together this implies that CNN appears to be more important in holding the structure of the centrosome together than does the γ -TuRC, and this is perhaps to be expected from the predicted coiled-coil nature of CNN.

It is clear that mitotic spindles can form and function in the absence of centrosomes. Repeated rounds of mitosis are known to take place in the absence of centrosomes in the unfertilized eggs of *Sciara* flies (de Saint Phalle and Sullivan 1998). Moreover, in *Drosophila* eggs derived from *polo* mothers, the four products of female meiosis are capable of undergoing many rounds of mitosis on acentriolar spindles (Riparbelli et al. 2000). These spindles strongly resemble the meiotic spindles of female *Drosophila* in which γ -tubulin cannot be detected by immunostaining at these spindle poles, even though it is apparently needed for spindle function (Tavosanis et al. 1997). The ability to build a functional spindle in *Xenopus* extracts in the absence of centrosomes is also well documented and requires minus end directed motors such as dynein to focus the poles (Heald et al. 1996). The consequences of removing centrosomes from cells that have robust checkpoints to monitor spindle assembly can vary, and could reflect either or both the cell line studied and exactly how the experiment was performed. Microsurgical removal of centrosomes has been reported to block future cycles of cell division (Maniotis and Schliwa 1991; Zhang and Nicklas 1995a,b). On the other hand, laser directed ablation of either one or both centrosomes did not prevent assembly of spindles that could successfully undertake anaphase (Khodjakov et al. 2000). The high mitotic index resulting from partial disruption of the centrosome in *dd4* mutants suggests a mitotic delay likely to result from activation of the spindle integrity checkpoint known to be functional in larval brain cells.

The distribution of the Asp following the apparent breakdown of the γ -TuRC gives insight into how these proteins might cooperate in microtubule nucleation. It is known that, following KI depletion of centrosomes, their ability to organize asters of microtubules can only be restored by supplying a complementary cytoplasmic extract that contains both the γ -TuRC and functional Asp protein (Moritz et al. 1998; Avides and Glover 1999). In wild-type cells, Asp forms a hemispherical cup-like structure on the face of the spindle microtubules suggesting that it is contacting the minus ends of these, and not the astral microtubules. Astral microtubules are not seen in *dd4* mutant cells at either the light or EM levels, and the spindle poles exhibit varying degrees of disorganization. Nevertheless, the Asp protein is invariably present at the spindle poles even in those extreme cases where individual bundles of microtubules are no longer held together at a single poorly focused pole. In such cases Asp appears at the very tips of these tubules as if it is providing some capping property to their minus ends.

It is difficult to compare the effects of *γ -tub23C* and *dd4* mutations upon the structure of the centrosome itself, as many of the antibodies used as reagents to iden-

tify specific centrosomal components were not available to the earlier study of Sunkel and colleagues (1995). Nevertheless, although the centrosome had abnormal morphology judged by the distribution of CP190 (Bx63 antigen) in the *γ -tub23C* mutant, the antigen was only noted as being at pole-like structures. Unfortunately, there are currently no known mutants of the *CP190* gene, and its function remains unknown. CP190 exists in a complex with CP60, and both proteins are known to be nuclear during interphase and move onto centrosomes at mitosis (Kellogg et al. 1995; Whitfield et al. 1995). The extent of interaction between these proteins and γ -tubulin is also unclear. Two complexes containing γ -tubulin have been purified from *Drosophila* embryos, the 3 MD γ -TuRC itself, and a smaller complex of 240 kD that appears to be a sub-unit of the larger one. The CP190–CP60 complex appeared not to be present in either of these γ -tubulin complexes from which it was separable by gel-filtration (Moritz et al. 1998). However, Raff and colleagues (1993) were able to detect low levels of γ -tubulin in the eluate from immunoaffinity columns constructed from antibodies to CP190 and CP60. This has led to the speculation that although these proteins may not assemble with each other in stoichiometric ratios, they may still show interactions, either on an affinity column in vitro or during centrosome assembly. Consistent with this is the observation that following loss of the majority of the γ -TuRC from the centrosome in *dd4*¹, some CP190 remains in the centrosome, whereas some dissociates and clusters in punctate arrays in the region of the spindle occupied by the condensed chromosomes. In this sense it may be obeying elements of a nuclear localization signal that directs its interphase location, the nuclear envelope undergoing incomplete breakdown during mitosis in *Drosophila* to form a fenestrated envelope around the spindle (Stafstrom and Staehelin 1985; Barbosa et al., unpubl.).

Despite the differences in fixation procedures, several aspects of the ultrastructure of the mitotic apparatus in *dd4* cells as seen by electron microscopy, such as the microtubule density, are concordant with our observations by immunofluorescence. Chromosomes are abnormally condensed and the number and density of spindle microtubules is greatly reduced in the mutant cells. The dispersion of the γ -tubulin, which we assume to be the primary consequence of the *dd4* mutations, is reflected by disorganization of the PCM and altered centriole morphology. Some γ -tubulin has previously been shown to be localized to the core of the centriole (Fuller et al. 1995), and inactivation of the γ -tubulin gene in *Paramecium* leads to inhibition of the duplication of the related structures, the basal bodies (Ruiz et al. 1999). The finding of fewer than four centrioles in the serial EM sections of some *dd4* mutant cells suggests a failure of centriole duplication. However, the failure to find centrioles at one of the poles in six *dd4* mutant cells suggests that centrosome separation is also dependent upon a functional γ -TuRC. This may be related to a function in correctly holding centrioles together, as mother and daughter centrioles are rarely perpendicular. The extent to

which other centrosomal components found principally in the PCM can contribute to structure of the centriole is not clear. Nonetheless, it is interesting in this context that an isoform of CNN expressed during spermatogenesis is localized both to the centrosomes and to the basal body and has been shown by mutational analysis to be required for the organization of the flagellar axoneme that develops from the spermatid basal body (Li et al. 1998).

Materials and methods

dd4 alleles used in this study

We have examined the three *dd4* alleles indicated in Table 1. *dd4*¹ males display the strongest phenotype with larvae dying in the early third instar stage of development and showing a very abnormal morphology of brain and associated imaginal discs. The allele *dd4*¹ was generated by EMS mutagenesis. *dd4*² males can reach late pupal stages but never eclose and their discs are also degenerated. The *dd4*² allele was generated by ENU (ethyl nitrosourea) mutagenesis (A. Carpenter, unpubl). *dd4*^S arose spontaneously in a cross of wild-type males and *mei-9* females (A. Schalet, pers. comm.). *dd4*^S mutant males normally die just after the eclosion but in dry cultures both males and females are viable although almost completely sterile. They show wings held up, absence of some bristles, and defects in abdominal segments, seen in other cell cycle mutants.

Molecular biology

Standard molecular biology techniques were employed following Ausubel et al. 1999 and Sambrook et al. 1989. The plasmid vectors pBluescript I and II (Stratagene) were used for all subcloning.

P-element-mediated germ-line transformation

A 9 kb *EcoRI* fragment from EDGP cosmid 22F12 was subcloned into the transformation vector pCaSpeR (Pirrota 1988) to give the rescue construct P[w⁺, E9]. A *SalI-EcoRI* fragment of ~5.6 kb was subcloned into the *EcoRI-XhoI* site of the transformation vector pW8 (Klemenz et al. 1987) to form the negative rescue construct P[w⁺, ES5.6]. Embryos of the genotype *w*¹¹¹⁸/*w*¹¹¹⁸; +/-; $\Delta 2-3(68C)/+$ were used for microinjection as described by Karess (1985).

Creation of a new dd4 allele by X-irradiation

Males carrying the P[w⁺, *ry*⁺]/E2 chromosome (Levis et al. 1985) aged 3–5 d were exposed to X-rays to achieve a dose of 40–80 Gy using a Torrex 150D X-ray machine (150 kV, 5 mA; Astrophysics Research Corporation) and then mated to *Basc* virgin females. The female progeny of this cross were then scored for loss of the w⁺ eye colour phenotype. All such mutants were rebalanced over *FM7*. From a total of 38,000 female progeny we obtained 21 w⁺ lines: 15 were viable, five were lethal, and one was male sterile. The lethal mutants were tested for their ability to complement the lethality of *dd4*^S. Heterozygous *xr* mutant/*FM7* virgin females were crossed to *dd4*^S males in which the Y chromosome carried the translocated duplication *y*⁺ *g*⁺ *na*⁺Y (12A8;12F; A. Schalet, pers. comm.). The lethal mutant *xr16* was not complemented by *dd4*^S. Because the *dd4* locus has not been genetically separated from *mus101* or *garnet* (Axton 1990, A. Schalet, pers. comm.), we tested complementation be-

tween *xr16* and *mus101* and *garnet* alleles. *xr16* complements the eye color phenotype of *g*⁺, the lethality of *mus101*sm, and the mutagen sensitivity phenotype of *mus101*^{D1}, *mus101*^{D2}, and *mus101*^{K451}. The mutagen sensitivity tests were carried out as described (Yamamoto et al. 2000). The mutant *xr16* was re-named to *dd4*^{xr16}.

Molecular localization of dd4

The position of the *dd4* locus was identified by a RFLP in the mutant *dd4*^{xr16}. DNA was extracted from *dd4*^{xr16}/*FM7*, *dd4*^S/*FM7*, *dd4*¹/*Binsn*, *dd4*²/*FM7*, Oregon R and *FM7* females, digested with *EcoRI* and analyzed in Southern-blot experiments. The 9 kb *EcoRI* genomic fragment (E9) immediately proximal to *garnet* was used as a probe. Of all *dd4* alleles tested, only *dd4*^{xr16}/*FM7* exhibits a RFLP in the E9 fragment.

Isolation of dd4 cDNAs

Three apparently identical *dd4* cDNAs were isolated from a 0–3 h embryonic library in λ gt10 (Poole et al. 1985) screened with the genomic *EcoRI* fragment E9. One of these, cDNA E912, is 1465 nucleotides long and encodes a homolog of the budding yeast Spc98 protein. However, it appeared to be incomplete as it lacked a 5' non-translated sequence and it was smaller than the Spc98 homologs reported in human and *Xenopus*.

Another cDNA, 8-1, was isolated from a 4–12 h λ ZAP embryonic cDNA library (Stratagene), probed with E912 cDNA. cDNA 8-1 is 2038 nucleotides long, and has an ORF of 501 amino acids. The coding sequence is followed by 533 nucleotides of 3' non-coding region, including the potential polyadenylation signal AATAAA. The sequence of a second cDNA isolated from this λ ZAP library overlapped cDNA 8-1 for positions 1036–2038. Even though the cDNA 8-1 is the longest cDNA isolated, it only extends 433 nucleotides upstream of cDNA E912. Thus, it is also unlikely to be complete.

Sequencing of the E9 genomic fragment

The E9 fragment was sequenced using internal primers designed specifically for its sequence and by creating a series of nested deletions using Exonuclease III (Promega). Sequencing reactions were performed using the ABI PRISM big dye terminator cycle sequencing kit (Applied Biosystems). The E9 genomic fragment is 9883 bp long. A search for ESTs in the *Drosophila* genome project revealed three different ESTs, named transcripts 1, 2, and *dd4* in Figure 1G. Transcript 1, corresponding to ESTs HL01073 and Z31921 is similar to sugar isomerases from different organisms. Transcript 2, corresponding to ESTs GH04786, LP03652, and LD46448, is similar to rat estradiol 17 β -dehydrogenase. The *dd4* sequence corresponds to ESTs LD43640, GM14553, and LD42379, as well as Dgrip91 (Oegema et al. 1999). The *dd4* coding sequence is 2769 bp long and is interrupted by four introns (Fig. 1F). Transcripts 1 and 2 are nested in intron 4.

Immunofluorescent staining of brains

Immunostaining brains from wild-type (Oregon R) or mutant third-instar larvae was carried out as described previously (Gonzalez et al. 1990) except that no taxol treatment was performed. *dd4* hemizygous male larvae were washed and dissected in 1 \times phosphate buffered saline (PBS). Their brains were fixed in 1 \times PBS 3.7% formaldehyde for 20 min brains were then permeabilized for 10 min in PBS 0.3% Tween and blocked for 1 h in PBS 0.1% Tween, 10% fetal calf serum. Microtubules were de-

tected with the rat anti- α -tubulin antibody, YL1/2 (Sera-Lab) diluted 1 : 50 and a FITC-conjugated anti-rat IgG antibody (Jackson Laboratories). Centrosomin was revealed with the rabbit CNN-specific antibody (Heuer et al. 1995) kindly provided by Thomas Kaufman, at a dilution of 1 : 1000. γ -tubulin was localized using the monoclonal antibody from clone GTU88 (Sigma) diluted 1 : 50. Anti-Asp was polyclonal rabbit serum Rb3133 (Saunders et al. 1997) diluted 1 : 50 and anti-CP190 was Rb188 (Whitfield et al. 1988). Secondary antibodies were either from Jackson Immunochemicals (FITC- and TexasRed-conjugated) or Sigma (TRITC-conjugated) and used according to the supplier's instructions. DNA was stained with TOTO3 (Molecular Probes) also according to the supplier's instructions. Preparations were visualized in a Bio-Rad 1024 confocal scanning head coupled to a Nikon Optiphot microscope.

Cytological analysis of larval central nervous system

Wild-type and mutant larval CNSs were fixed and squashed in aceto-orcein, and whole-mount preparations of the larval CNS were carried out as described previously (Inoue and Glover 1998). The specimens were examined using phase-contrast optics of Nikon Microphot-FX in conjunction with a Nikon FX-35A photographic camera.

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Mutation of a *Drosophila* gamma tubulin ring complex subunit encoded by *discs degenerate-4* differentially disrupts centrosomal protein localization

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